Characterization of the Structural and Chemical Properties of Copper Chelators in Seawater

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LONG-TERM GOALS

The long-term goal is to obtain a comprehensive understanding of copper chemistry and bioavailability in seawater, at the molecular level. We are particularly interested in the relationship between water chemistry and biological effects of copper. This information can be used for accurate assessments of the impacts of Cu introduced to harbors by human activities. Such information may also be useful in the development of accurate and economical strategies to detect and remove Cu and other contaminants from waste. Results can be used by dischargers, like the US Navy, and regulators, including the EPA and local agencies, to make informed decisions about managing Cu inputs into harbors and other receiving waters.

OBJECTIVES

The primary objectives of this project relate to naturally occurring Cu binding ligands. Our work, and that of others, shows that these ligands control the variability in Cu bioavailability in many coastal waters. We seek to learn more about the chemical properties of these poorly characterized substances. Current titration methodologies provide information about binding constants and concentrations only. Structural information is necessary to validate hypotheses about sources and sinks (with a view to modeling variability) and to identify compounds with unique properties of relevance to the navy.

APPROACH

The approach has been to focus on chelators produced by ubiquitous marine phytoplankton and bacteria, rather than chelators actually in the water column, because they can be produced at much higher concentrations. In the past, we have made the case that some organisms may be important sources, such as the marine cyanobacterium *Synechococcus*. Cu stressed *Synechococcus* cultures produce a chelator with binding characteristics similar to the strongest ligands found in the water

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column. We hypothesized that this ligand is produced as a detoxification mechanism for Cu (Moffett and Brand, 1996).

We have combined measurements of the ligand concentration and thermodynamic properties by cathodic stripping voltammetry, with a several separation schemes to isolate ligands for HPLC analysis.

WORK COMPLETED

The following tasks were completed this year. We surveyed a more genetically diverse range of cyanobacteria than in previous years, including halotolerant and freshwater strains, for strong ligand production. We also developed several strategies for precolumn derivatization of metal binding ligands, focusing on functional groups that we expected to find. We developed protocols to analyze these derivatives by electrospray mass spectrometry, and applied our protocols to model ligands and natural samples.
We have been focusing on thiols, in particular an electroactive compound measured in the water column by other workers and attributed to glutathione.
We have studied the reaction kinetics of the unknown compound in comparison to glutathione with oxygen and transition metals as well as the effect of other parameters like pH and light.

The other major task completed was publication of ONR sponsored research. Nine ONR sponsored papers have been published or submitted since last year's report.

RESULTS

Last year we developed protocols for optimizing production of *Synechococcus* chelators as a function of growth rates, leading to ligand production at levels 100x greater than levels of the strongest ligands in natural waters. However, these levels are still much lower than comparable concentrations of siderophores (Fe binding ligands) produced by bacteria, which have been isolated and characterized. In order to produce more material, we carried out a much wider survey of *Synechococcus* clones, including some freshwater and halotolerant species that are genetically quite distinct from the marine species we had studied before. Previously, all strains we had studied produced strong chelators in response to Cu stress. However, the wider survey revealed that many coastal strains make no chelators at all, evidently having other strategies for detoxification. An interesting generalization has emerged from the study. All of the strains that make the strong ligand contain the light harvesting pigment phycoerythrin (the cultures are pink). All of the strains that don't make the ligand contain phycocyanin (the cultures are green). So far we have no explanation for this observation.

Cathodic stripping voltammetry performed on *Synechococcus* culture media revealed a large peak at –0.63 V (vs. Ag/AgCl). This peak has been observed by others in seawater and eukaryotic cultures and attributed to glutathione. However, many thiol compounds produce a peak at this potential besides glutathione. We investigated the behavior of this peak in the presence of copper and zinc and as a function of pH. We observed a pH dependent shift in potential identical to glutathione. However, addition of Cu or Zn to glutathione results in a disappearance of the peak and appearance of Cu and Zn peaks at different potentials. For the compound in our cultures, the thiol peak disappears but no complex peaks form. This suggests that the compound reacts with Cu and Zn but does not form a complex or that complexes are formed but do not adsorb onto the electrode. We favor the latter interpretation.

We do not know if the peak at –0.63V contains the strong ligand. It is consistent with work we reported last year on the determination of half wave potentials for Cu complexes in *Synechococcus* cultures that suggested Cu(I) complexes with thiols. This year, we titrated cultures with Cu(I) rather than Cu(II). We reasoned that Cu(II) might simply oxidize thiol ligands (producing Cu(I)). In contrast, addition of Cu(I) would lead to stoichiometric production of a Cu(I) thiol complex. However, results for Cu(I) and Cu(II) were identical despite stringent precautions to exclude oxygen.

Thus we are not sure if the "thiol" peak at –0.63V is the strong Cu ligand. However, we have decided to work on identifying it in order to answer this question conclusively, and because the compound may contribute to the peak at –0.63V measured by other workers in many marine environments and attributed to glutathione.

Santschi and coworkers have used thiol-derivatization coupled with HPLC to determine thiols in seawater and have detected glutathione, albeit at lower concentrations than workers who use the "thiol" peak detected by voltammetry. This year we have utilized derivatization as well, coupled with electrospray mass spectrometry (ESMS) of the resultant adducts. However, rather than derivatizing the thiol group, we used fluorescamine, a derivatizing agent for primary amines that is widely used for peptide analysis. This reagent would derivatize peptide thiols, like glutathione, but could also derivatize non-thiol peptides in the sample that might also be important chelators. Results show that fluorescamine is a highly effective derivatizing agent for glutathione and other naturally occurring chelators like desferrioxamine. In cultures derivatization produces several peaks. However, cultures containing strong Cu ligands have a single peak that is much larger than the rest. ESMS analysis suggests that this is a discrete compound. We are actively working on further fragmentation of the parent ion to determine whether this peak is a thiol peptide.

We are also collaborating with Beth Ahner (Cornell) who has a thiol derivatization protocol. Preliminary evidence suggests that *Synechococcus* cultures contain a thiol that does not co-elute with any of her thiol standards.

IMPACT/APPLICATIONS

Successful characterization of this material could lead to new insight into the sources and chemistry of Cu ligands in seawater. Potential applications could arise if we identify a new class of chelators selective for Cu that could be used in remediation.

TRANSITIONS

None recognized at this time.

RELATED PROJECTS

Moffett is collaborating with Richard Thompson (U. Maryland) who is supported with 6.2 funds to develop an in situ fiber optic biosensor for Cu. Moffett is assisting Thompson with calibration and field deployment. Moffett also works with Brian Palenik(Scripps) on a project to study the relationship between Cu chemistry in seawater and the production of a highly Cu specific cell-surface binding protein by marine diatoms. Palenik has developed an antibody to this protein which may be an excellent in situ indicator of metal stress.

PUBLICATIONS

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